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| APPLICATION NO./ CONTROL NO. | FILING DATE | FIRST NAMED INVENTOR / PATENT IN REEXAMINATION | ATTORNEY DOCKET NO. |
|---|---|---|---|

| EXAMINER |
|---|

| ART UNIT | PAPER |
|---|---|
| | 18 |

DATE MAILED:

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PTO 2003-1424

Japan, PCT
WO99/38991

# METHOD FOR TRANSFERRING GENE INTO GERM CELL
[Seishoku Saibo Eno Idenshi Donyuho]

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UNITED STATES PATENT AND TRADEMARK OFFICE
Washington, D.C. January, 2003

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|---|---|---|
| Country | : | Japan |
| Document No. | : | WO99/38991 |
| Document type | : | PCT |
| Language | : | Japanese |
| Inventors | : | Mitsuhiro Ueno, Haruko Konishi, Mio Morishita, Atsushi Yuki, Kiyozo Asada, and Ikunoshin Kato |
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| IPC | : | C 12 N 15/86<br>A 01 K 67/027<br>C 12 N 5/10 |
| Application date | : | January 20, 1999 |
| Publication date | : | August 5, 1999 |
| Foreign Language Title | : | Seishoku Saibo Eno Idenshi Donyuho |
| English Title | : | METHOD FOR TRANSFERRING GENE INTO GERM CELL |

# Method for Transferring Gene into Germ Cell

## Field of the invention

The present invention pertains to a method for transferring a gene into germ cells. More specifically, the present invention pertains to a method for transferring a gene to germ cells of a vertebrate, which is useful for medica, pharmaceutical, farming fields, etc., and a method for preparing a transgenic animal using the germ cells to which a gene is transferred by said method.

## Background of the invention

The development of recent genetic engineering techniques, animals in which genes are modified by the transfer of a foreign gene or the deficiency of a specific gene, that is, transgenic animals have been able to be prepared. The transgenic animal preparation technique is used as an experimental method for elucidating the functions of genes and also used as a means for obtaining pathologic model animals for being used in evaluating the performances of medical supplies and livestock having

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*Numbers in the margin indicate pagination in the foreign text.

excellent characteristics.

As the most general method for preparing the transgenic animals, a DNA is directly injected into a fertilized egg by microcomputer, etc., and the fertilized egg is transplanted into temporary parents, and a grown individual is obtained (Gordon, J.H. et al., Proceedings of the National Academy of Science of the USA, Vol. 77, pp.7380-7384 (1980)). In this method, in addition to processing of the in vitro fertilized egg, a very precise work of the DNA injection into the fertilized egg is required, and its success is expected only by the hands of a skilled researcher. Also, the DNA injected is not aggressively integrated into a chromosome DNA, the ratio in which an individual carrying a foreign gene is obtained is low.

Therefore, in order to reliably achieve the purpose, a number of fertilized eggs must be processed, so that problems such as work load and animal purchase cost for sampling the fertilized eggs are generated. For this reason, the following various methods have been considered and attempted.

In order to effectively integrate a foreign gene onto a /2
chromosome, an attempt of introducing the foreign gene into an initial embryo by a retrovirus vector is reported (Bowen, R.A. et al., Molecular Reproduction and Development, Vol. 40, pp.386-390 (1995)).

Furthermore, an attempt of introducing a foreign gene into an embryo by injecting a mixture of basic polyamide and plasmid into the vein of a pregnant female mouse is also reported (Terada

et al., Nature Genetics, Vol. 9, pp.243-248 (1995)).

On the other hand, a method for preparing a transgenic animal based on the principle in which a genetically modified sperm is prepared first and a fertilized egg is then prepared by using it is also attempted. For example, a method that kills testicular cells by a physical or chemical treatment and transplants genetically modified spermatogonia into a seminiferous tubule is reported (Brinster R.L. et al., Proc. Natl. Acad. Sci. USA, Vol. 91, pp.11303-11307 (1994) and Nature Medicine, Vol. 2, pp.693-696 (1996)).

Also, an attempt that coprecipitates a ring-shaped or straight chain-shaped plasmid DNA containing a foreign gene by calcium phosphate, doses it into testes by a ribosome injection is also made (Tada et al., Animal Biotechnology, Vol. 5, pp.19-31 (1994) and Takahashi et al., Summary of Japanese Livestock Society, 2x-8 (1996)).

The above-mentioned methods of "Binster et al." and "Bowen et al." mainly consider the acquisition of a genetically modified individual with high efficiency. However, since these techniques require high-level experimental techniques such as maintenance of an initial embryo, gene introduction by a virus, and cell transplantation to a testicular seminiferous tubule and expensive facilities for enabling the techniques, they cannot be applied by ordinary experimental facilities.

Furthermore, the methods of "Tada et al.," "Takahashi et al," and "Terada et al." are simple methods without requiring the

above-mentioned facilities, however a foreign gene dosed is not aggressively integrated on a chromosome of germ cells as a target. Therefore, even if its existence is transiently confirmed, the possibility of being transferred to offsprings seldom exists. In other words, the above techniques are not suitable for a method
for obtaining germ cells in which a biologically functional /3
intended gene is integrated and a transgenic animal by using said cells as an origin.

<u>Purpose of the invention</u>

The purpose of the present invention is to provide a simple method for obtaining germ cells in which an intended foreign gene is integrated and a vertebrate for holding said cells.

Also, another purpose of the present invention is to provide a method for preparing a genetically new vertebrate system utilizing the above-mentioned germ cells or vertebrate.

<u>Outline of the invention</u>

These inventors discovered that in a gene introduction into testicular cells that inoculates a recombinant virus into testes of a vertebrate, gene-introduced testicular cells could be simply obtained with good efficiency by the combination of the virus inoculation and the process for reducing the number of testicular cells. Then, the present invention was completed.

In other words, a first invention of the present invention pertains to a method for transferring a gene to testicular cells of a vertebrate and is characterized by including a process that

reduces the number of testicular cells and a process that inoculates a recombinant virus having a foreign gene into the testes. As a method for reducing the testicular cells, chemical method, physical method, biological method, etc., can be used, and in particular, a chemical method using an alkylating agent is appropriate. Also, the recombinant virus is preferably inoculated into the testes at a recovery stage of the testicular cells.

A second invention of the present invention pertains to a vertebrate in which a foreign gene is transferred to the testicular cells and is characterized by transferring a foreign gene by the method of the first invention.

A third invention of the present invention pertains to testicular cells into which a foreign gene is introduced and is characterized by being obtained from the vertebrate of the second invention.

Furthermore, a fourth invention of the present invention pertains to a vertebrate into which a foreign gene is introduced and is characterized by being obtained by mating of the vertebrate of the second invention or an artificial fertilization

using the testicular cells of the third invention. /4

Detailed explanation of the invention

The method for transferring a gene to testicular cells of the present invention uses a recombinant virus as a vector. It is desirable for the virus vector usable in the present invention

to have an ability of integrating an intended gene onto a chromosome of target cells, and for example, retrovirus vector, human immunodeficiency virus (HIV) vector, adeno-associated virus vector, etc., can be used, though it is not particularly limited. Furthermore, it is desirable for the virus vector of the present invention to be deficient for replicability so that an unlimited infection may not be repeated.

In the method for transferring a gene of the present invention, a well-known replication-deficient retrovirus vector can be used. For example, retrovirus vectors such as MFG (ATCC No. 68754), α-SGC (ATCC No. 68755), pLRNL (Virology, Vol. 171, pp.331 (1989)), and pBabe (Nucleic Acids Research, Vol. 18, pp.3587-3596 (1990)) or these modified vectors can be used, though it is not particularly limited. Also, these vectors can prepare a virus supernatant fluid containing virus particles in which said vectors are packaged by using well-known packing cell lines, for example, cell lines of PG13 (ATCC CRL-10686), PG13/LNc8 (ATCC CRL-10685), PM317 (ATCC CRL-9078), GP+E-86 (ATCC CRL9642), GP+envAm-12 (ATCC CRL9641), etc.

The foreign gene being introduced into germ cells by the method of the present invention is not particularly limited, and a desired optional gene being introduced may be used. For example, genes for coding oxygen and other proteins, genes for coding functional nucleic acid molecules such as anti-sense nucleic acid and ribozyme, and decoy can be introduced. The origin of these genes is not particularly limited, and genes

ascribed to the same organisms as the germ cells into which said genes are introduced, genes ascribed to different kinds of organisms, chemically synthesized genes, or these combinations may be adopted. Also, adjusting elements such as appropriate promotor, terminator, enhancer for adjusting the manifestation may be added to these genes. These genes are integrated into the above-mentioned virus vectors and used in the present invention.

The method for transferring a gene of the present invention includes the process that inoculates a recombinant virus containing a foreign gene being introduced into testes. The virus inoculation is carried out by injecting into testes, using a syringe under anesthesia, for instance, without a surgical operation such as celiotomy.

In the virus infection into cells, it is known that the number of target cells and the number of virus particles, that is, the ratio of the virus to the titer are factors having a large influence on the infection efficiency. For example, these inventors carried out a natural mating for 5 weeks that made normal female mice of 8 weeks of age live together at one to one from Monday to Friday per week, using 10 male mice in which a foreign gene-integrated retrovirus ($1 \times 10^4$ cfu/ml) was injected at 50 $\mu$l each into both testes. When 534 produced mice in total obtained from these 50 female mice were genetically analyzed by a PCR method, the produced mice having the foreign gene used were not recognized.

In order to solve this problem, a high-titer recombinant virus is preferably inoculated, and the titer of the recombinant virus is determined by a producer cell for producing said virus. In actuality, the construction of the producer cell for producing the retrovirus vector with a high titer is not easy. In the method for transferring a gene of the present invention, this problem was solved by combining the process for reducing the number of testicular cells.

The testicular cells described in this specification mean germ cells existing in testes. For example, a gene is transferred to the testicular cells such as spermatogonia, primary spermatocytes, spermatids, and sperms by the method of the present invention.

As the method for reducing the testicular cells, chemical method, physical method, and biological method can be used alone or in combination. The chemical method means a method for reducing testicular cells by dosing a chemical substance, and for example, DNA synthesis inhibitor, RAN synthesis inhibitor, DNA polymerase inhibitor, mitotic inhibitor, alkylating agent, oral contraceptive, etc., can be used. As the physical method, for example, the irradiation of X-rays and gamma rays can be used. Also, as the biological method, for example, proteins related to the multiplication and replication of cells can be utilized. /6
With the above-mentioned various methods, the number of testicular cells can be reduced. In the present invention, it is preferable to reduce the testicular cells by the chemical method

that easily controls the operation without special apparatus and operation.

As the method for chemically reducing the testicular cells, for example, there is a method using an alkylating agent. As the alkylating agent usable in the method of the present invention, for example, mitomycin C, nitrogen mustard-N-oxide, TESPA, merphalan, carbokuwon[transliteration], nitrosourea derivative, cyclophosphamide, busulphan, etc., are mentioned.

The method for transferring a gene of the present invention includes the process that reduces testicular cells to a recoverable extent in a state before treating. Mature cells are drained out in the testes by the above-mentioned reduction treatment of the testicular cells. However, the remaining spermatogonia are actively divided and start to multiply, and the number of cells is recovered to a state before treating after a lapse of fixed period. This phenomenon can be confirmed by the observation of testes with time, the microscopic observation of the structure segments of testes, the analysis of the cell period by a FACS (Fluorescence Activated Cell Sorting, and the in vivo mating experiment. In other words, with such an experiment, the correlation between the reduction treatment of the testicular cells and the reduction to recovery of the number of cells can be understood in detail.

If a recombinant virus containing a foreign gene is inoculated in the testes in which the number of cell is reduced in this manner, a virus infection to the testicular cells is

caused with high efficiency, and along with the recovery of the number of testicular cells, spermatogonia, primary spermatocytes, spermatids, and sperms into which the foreign gene is introduced are accumulated in the testes. Preferably, after the recovery stage where the remaining cells are actively multiplied is selected, a virus is dosed. It is known that the retrovirus being frequently used as a vector in the gene transfer to the cells infects the cells at a mitotic period, and the recovery period is an especially favorable environment for the infection of said virus.

With the incubation of the recombinant virus with a high titer, the efficiency of the gene transfer can be further raised. This can be achieved by selecting a packing cell having a high-titer virus producibility and preparing a virus supernatant fluid. Also, a virus enriched by an appropriate method may be used. For example, VSV-G vector (Proc. Natl. Acad. Sci. USA, /7
Vol. 90, pp.8033-8037 (1994)) as a retrovirus vector called a pseudo type can enrich virus particles by a centrifugal separation.

The present invention provides a method for transferring a foreign gene to simple vertebrate germ cells without a surgical operation and processing in vitro germ cells. With the use of the present invention, a male vertebrate individual for retaining the germ cells to which the foreign gene is transferred. Furthermore, the male vertebrate individual obtained is mated with a female individual, so that a fertilized egg ascribed to

the germ cells to which the gene is transferred and the vertebrate individual generated from said fertilized egg can be obtained. Also, in obtaining said fertilized egg, methods other than the mating can be used. For example, the testicular cells such as sperms are sampled from the above-mentioned male vertebrate individual and sterilized with an egg in a test tube by an artificial fertilization, so that a fertilized egg can also be prepared. The fertilized egg prepared by the artificial fertilization is transplanted to an appropriate temporary parent, it can be generated as an animal individual.

In the animal individual obtained in this manner, an individual in which the foreign gene is transferred onto one chromosome of the cell, that is, the gene is modified (hetero individual) is included. For such an individual, whether or not the foreign gene is included in the gene is investigated by a well-known method such as PCR method or hybridization method and can be selected. A homo individual, that is, an individual in which the gene on both chromosomes is modified is selected from the offspring being obtained by mating of these individuals is selected, so that a transgenic animal in which the gene introduced and the characteristic ascribed to said gene can be obtained.

Also, the present invention can be applied to all kinds of animals other than human being, and for example, the present invention can also be applied to livestock and poultry in addition to rodent animals (for example, mice, rats, etc.) being

frequently used for researches. With the use of the method of the present invention, a transgenic animal having a useful characteristic as an experimental animal or an excellent characteristic as a livestock can be simply, inexpensively prepared.

Also, the male vertebrate individual in which an intended foreign gene is introduced into the testicular cells by the method of the present invention produces sperms in which said gene is integrated onto its chromosome. Therefore, said method is /8 expected as a method for preparing breeding cow and breeding horse for producing ponies and calves with a high value added. Offsprings having said gene can be produced by mating using the male.

<u>Brief description of the figures</u>

Figure shows data of testicular cells of a busulphan-dosed mouse analyzed by the FACS. In the figure, A, B, and C show analysis results of the testicular cells after 1, 3, and 5 weeks from the busulphan dosage.

Figure 2 shows the generative power of a busulphan-dosed male mouse. The pregnancy rate of the figure shows the ratio of pregnant female mice. Also, in the figure, A shows the result of a control group, and B and C shows the results of mice to which 20, 30, and 40 mg/kg busulphan are respectively dosed.

Figure 3 shows the region of vGL2 virus gene being integrated into a cell chromosome being infected, which is included in a plasmid pGL2. In the figure, LTR is a LTR (Long

Terminal Repeat) ascribed to a Moloney mouse leukemia virus, SD is a splice donor part, $\phi$ is a packaging signal, GMV is an initial enhancer promotor ascribed to a cytomegalovirus, GFP is a green fluorescence protein gene, SA is a splice acceptor part, Neo is a neomycin phosphotransferase gene, SV40ori is a replication start point ascribed to SV40, and pBR322ori is a replication start point ascribed to pBR322.

Figure 4 shows the results of the manifestation of GFP in testicular cells of a mouse inoculated with vGL2 virus analyzed by the FACS.

Figure 5 shows the results of the manifestation of GFP in sperms sampled from the epididymis of the mouse inoculated with vGL2 virus analyzed by the FACS.

Application examples

Next, the present invention is explained in further detail by application examples, however the present invention is not limited to these application examples.

Application Example 1

Variation of testicular cells of a mouse by an intraperitoneal administration of busulphan

(1) Influence on the weight of testes /9

5 mg/kg, 10 mg/kg, 20 mg/kg, and 40 mg/kg busulphan (1,4-butanediol dimethane sulfonate, made by Wako Pure Chemical Industries, Ltd.) was dosed into the peritoneal cavity of MCH (ICR) male mice of 4 weeks of age. After dosing, the testes of the mice were extracted on the first week, the second week, the

third week, the fourth week, and the fifth week, and the weight was measured and measured with the weight of the testes of mice to which a physiological saline solution was dosed as a control group.

As a result, as shown in Table I, the decrease of the weight was observed in the mice to which 20 mg/kg and 40 mg/kg busulphan was dosed. Furthermore, in the mouse to which 20 mg/kg busulphan was dosed, it was confirmed that the weight tended to be recovered on the fifth week after dosing.

Table I

Influence of busulphan dosage on the weight of testes of mice

| ブサルファン投与量 (mg/kg) | 薬剤投与後の時間 (週) | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| 対照 | 93.9 mg | 129.5 mg | 123.4 mg | 140.8 mg | 145.6 mg |
| 5 | 98.2 mg | 124.5 mg | 105.0 mg | 114.7 mg | 97.4 mg |
| 10 | 75.8 mg | 113.3 mg | 106.1 mg | 112.4 mg | 115.1 mg |
| 20 | 101.5 mg | 96.3 mg | 78.8 mg | 71.4 mg | 87.9 mg |
| 40 | 100.8 mg | 89.0 mg | 46.9 mg | 45.6 mg | 34.2 mg |
| マウス週齢 | 5週齢 | 6週齢 | 7週齢 | 8週齢 | 9週齢 |

1. Amount of busulphan dosed (mg/kg)
2. Time (week) after dosing the drug
3. Control group
4. Mouse age
5. 5 weeks of age
6. 6 weeks of age
7. 7 weeks of age
8. 8 weeks of age
9. 9 weeks of age

(2) Influence on the body weight

10 mg/kg, 20 mg/kg, 30 mg/kg, and 40 mg/kg busulphan were dosed into the peritoneal cavity of the MCH (ICR) male mice of 4 weeks of age, and the body weight change until 7 weeks after dosing was measured and compared with the body weight of the

control mice to which a physiological saline solution was dosed. Also, five mice per group were used in each dosage group and the control group.

As a result, as shown in Table II, in the amount dosed in these ranges, the busulphan had no influence on the body weight of the mice. Also, no change was recognized in the appearance of the mice.

Table I/II 0

Influence of busulphan dosage on the body weight of mice

| マウス週齢 | 投与後時間(週) | 薬剤投与量 | | | | |
|---|---|---|---|---|---|---|
| | | 対照 | 10 mg/kg | 20 mg/kg | 30 mg/kg | 40 mg/kg |
| 4 | 0 | 24.8 ± 0.3 | 25.6 ± 0.7 | 24.0 ± 0.6 | 23.9 ± 0.6 | 23.9 ± 0.6 |
| 5 | 1 | 31.1 ± 1.4 | 31.2 ± 1.2 | 未測定 | 未測定 | 未測定 |
| 6 | 2 | 32.8 ± 2.0 | 33.8 ± 1.9 | 未測定 | 未測定 | 未測定 |
| 7 | 3 | 35.4 ± 2.1 | 35.6 ± 1.6 | 未測定 | 未測定 | 未測定 |
| 8 | 4 | 36.0 ± 2.1 | 36.3 ± 1.3 | 36.7 ± 1.4 | 34.7 ± 1.5 | 36.2 ± 0.9 |
| 9 | 5 | 35.2 ± 2.5 | 37.3 ± 1.6 | 36.7 ± 1.6 | 35.4 ± 1.3 | 36.3 ± 0.7 |
| 10 | 6 | 34.9 ± 2.3 | 36.5 ± 1.3 | 36.1 ± 1.6 | 34.4 ± 1.7 | 35.6 ± 1.5 |
| 11 | 7 | 35.1 ± 2.2 | 36.1 ± 1.0 | 36.8 ± 2.3 | 34.7 ± 1.3 | 36.8 ± 2.0 |

1. Mouse age
2. Time (week) after dosing
3. Amount of drug dosed
4. Control group
5. Unmeasured
6. Shown by the average weight ± standard deviation (unit: g) of five mice per group.

(3) Observation results of testes and tissue segments of epididymis by microscope

20 mg/kg, 30 mg/kg, and 40 mg/kg busulphan were dosed in the peritoneal cavity of MCH (ICR) male mice of 4 weeks of age. After dosing, the testes of the mice and the epididymis were extracted on the 10th week, and tissue segments were prepared. These segments were dyed with HE (hematoxylin and erosin stain), and observed by a microscope. The state of the tissues and the cells was compared with those of the control mice to which a physiological saline solution were dosed. Also, five mise per group were used in each dosage group and the control group.

As a result, as shown in Table III, both the testes and the epididymis were recovered to a state similar to the control mice in the dosage group of 20 mg/kg and 30 mg/kg.

Table III

Microscopic observation results of tissue segments of testes and epididymis of busulphan-dosed mice

| 薬剤投与量 | 顕微鏡観察 | | | | 判定（回復状態） |
|---|---|---|---|---|---|
| | 精巣内細胞 | | 精巣上体精子 | | |
| | 精細管 | 精原細胞 | 上体頭部 | 上体尾部 | |
| 対照 | 充実 | 充実 | やや充実 | 充満 | 正常 |
| 20 mg/kg | 充実 | 充実 | やや充実<br>一部中空管有 | 充満 | 回復 |
| 30 mg/kg | やや充実<br>剥離管有 | 欠失～<br>やや充実 | やや充実<br>一部中空管有 | 微量～充満 | 回復 |
| 40 mg/kg | やや充実<br>剥離管有 | 欠失～<br>やや充実 | やや充実<br>一部中空管有 | 空洞化～<br>やや充満 | 未回復<br>一部回復有 |

1. Amount of drug dosed
2. Microscopic observation
3. Decision (recovery state)
4. Testicular cells
5. Epididymis sperms
6. Seminiferous tubule
7. Spermatogonia
8. Epididymis head
9. Epididymis tail
10. Control group
11. Complete
12. Slightly complete
13. Full
14. Normal

15. Slightly complete

    Partial hollow tube

16. Recovered

17. Slightly complete

    Peeled-off tube

18. Deficient to slightly complete

19. Slightly complete

    Partial hollow tube

20. Infinitesimal amount to full

21. Slightly complete

    Peeling-off tube

22. Deficient to slightly complete

23. Slightly complete

    Partial hollow tube

24. Cavity to slightly full

25. Unrecovered

    Partial recovery

26. Decided based on the microscopic observation results of the tissue segments of the testes and the epididymis.

(4) Analysis of testicular cell groups of an intraperitoneal administration of busulphan by FACS

In MCH (ICR) male mice of 4 weeks of age in which 6 mice were one group, 20 mg/kg and 30 mg/kg busulphan were dosed, and a physiological saline solution was dosed into the peritoneal cavity of a control group. After dosing, on the first week, the second week, the third week, the fourth week, the fifth week, and the sixth week, testes were extracted from each one mouse of each group, and the testicular cells were treated according to the following operation. First, after removing the epidermis of the testes, Collagenase I (made by Gibuko Co.) with a final concentration of 1 mg/ml was added to it and treated at 37°C for 15 min, washed, and cell bulks were separated using 25% trypsin/EDTA (made by Gibuko Co.) 1 ml. Next, the cell bulks of 10 $\mu$ or more were removed by a filter, and the cells were fixed with 1% paraformaldehyde and then fixed with 70% ethanol. The cells were washed with phosphoric acid buffer physiological saline solution (PBS), suspended in the PBS, treated with 2 mg/ml RNaseA (made by Sigma Co.), and DNA-dyed with 0.05 mg/ml propidium iodide (made by Sigma Co.). Right before measuring, the cell bulks were removed through a mesh of 30 $\mu$, and the DNA content of the cells was analyzed at an excitation wavelength of 488 nm and a measurement wavelength of 564-606 nm by using FACSVantage (made by Vecton Dixon Co.).

According to the FACS analysis, the spermatogonia and the

primary spermatocytes at G2 stage and M stage in which the amount of DNA is 4n, the spermatogonia and the secondary spermatocytes at G1 group in which the amount of DNA is 2n, the spermatids and the sperms in which the amount of DNA is n, and the number of /12
cells in which the amount of DNA is smaller than that and an apoptosis is caused can be attained. Table IV shows the values attained by the analysis data. Also, Figure 1 show data of the FACS obtained for the testicular cells after 1, 3, and 5 weeks of the mice to which 20 mg/kg busulphan was dosed as a representative example.

Figure 1 shows that most of the testicular cells cause the apoptosis on the third week after dosing, while the ratio of the number of cells of 2n is increased on the fifth week. Also, from the results shown in Table IV, it is clarified that the total number of testicular cells is smallest after about 4 weeks from the busulphan dosage, the regeneration of the cells is started thereafter, and the number is increased first from the cells of 2 n in the regeneration process of the cells.

Table IV

Variation of testicular cell species of mice after an intraperitoneal administration of busulphan

| ブサルファン投与量 | ブサルファン投与後期間 | 細胞数 (×10⁵/精巣) | | |
|---|---|---|---|---|
| | | n | 2n | 4n |
| 対照 | 0週目 | 53.4 | 12.4 | 7.8 |
| | 1週目 | 108.6 | 17.7 | 18.3 |
| | 2週目 | 97.8 | 10.7 | 9.4 |
| | 3週目 | 87.6 | 8.9 | 4.3 |
| | 4週目 | 132.8 | 20.8 | 13.3 |
| | 5週目 | 49.8 | 206.1 | 21.4 |
| | 6週目 | 93.5 | 376.0 | 61.3 |
| 10 mg/kg | 0週目 | 53.4 | 12.4 | 7.8 |
| | 1週目 | 72.2 | 11.7 | 4.7 |
| | 2週目 | 110.0 | 12.7 | 3.6 |
| | 3週目 | 64.0 | 13.2 | 1.6 |
| | 4週目 | 36.6 | 11.6 | 3.8 |
| | 5週目 | 4.3 | 47.4 | 9.3 |
| | 6週目 | 4.7 | 153.4 | 17.0 |
| 20 mg/kg | 0週目 | 53.4 | 12.4 | 7.8 |
| | 1週目 | 78.4 | 10.1 | 4.7 |
| | 2週目 | 67.6 | 7.8 | 0.7 |
| | 3週目 | 22.8 | 7.6 | 0.7 |
| | 4週目 | 4.7 | 7.4 | 0.6 |
| | 5週目 | 2.6 | 10.2 | 6.7 |
| | 6週目 | 6.1 | 33.7 | 14.4 |

数値は2匹のマウスについての各細胞数の平均値を示した.

n : 精細胞および/または精子

2n : G1 精祖細胞および/または二次精母細胞

4n : G2,M 期の精祖細胞および/または一次精母細胞

1. Amount of busulphan dosed
2. Time after dosing busulphan
3. Number of cells (x $10^5$/testes)
4. Control group
5. 0 week
6. First week
7. Second week
8. Third week
9. Fourth week
10. Fifth week

11. Sixth week

12. The results showed the average values of the number of each cell for two mice.

    n: Spermatids and sperms

    2n: Spermatogonia at G1 stage and/or secondary spermatocytes

    4n: Spermatogonia at G2 and M stage and/or primary spermatocytes

(5) Reduction of the generative power of male mice by an intraperitoneal administration of busulphan and determination of recovery timing /14

20 mg/kg and 30 mg/kg busulphan were dosed into the peritoneal cavity of MCH (ICR) male mice at 4 weeks of age in which 6 mice were one group, and from the first week to the tenth week after dosing, the male mice were naturally mated with different female mice for each week for 5 days from Monday to Friday. With the investigation of the pregnancy rate of the female mice,, the generative power of the busulphan-dosed male mice and a control group to which a physiological saline solution was dosed was compared.

As a result, as shown in Figure 2, in the groups in which 20 mg/kg and 30 mg/kg busulphan were dosed into the peritoneal cavity, the decrease of the generative power (one the fourth to seventh weeks after the busulphan dosage) and its recovery (on

the eighth week after the busulphan dosage) were recognized. In the mice, the spermatogonia become the spermatids through the primary spermatocytes, and about 3-6 weeks are required until mature sperms. In the microscopic observation results of the tissue segments shown in the above-mentioned Application Example 1-(3) and (4) and the analysis results of the testicular cell group change of the FACS, the recovery of the testicular cells after the busulphan dosage is observed from the fifth week after dosing the drug. The recovery of the generative power of the male mice was recognized with a delay of 3 weeks from it. This facts well agreed with the time required until the mature sperms were formed from the spermatogonia.

Application Example 2

Introduction of a foreign gene into the testicular cells of mice and manifestation

(1) Construction of recombinant retrovirus

A plasmid pGreen Lantern-1 (made by Life Tech Oriental Co.) containing a gene for coding a green fluorescence protein (GFP) in which a codon was optimized for human beings and an amino acid being coded at 65th from N terminal was substituted by threonine (S65T) was digested by SspI (made by Takara Shuzo Co., Ltd.) and electrophoresed with 1% agarose gel, and a DNA fragment corresponding to about 1.9 kb was recovered. On the other hand, a plasmid pZIP-NeoSV(X)I (Cell, Vol. 37, pp.1053-1062 (1984)) was digested with BamHI (made by Takara Shuzo Co., Ltd.), and the terminal was smoothed using a DNA blanching kit (made by Takara

Shuzo Co., Ltd.). The phosphoric acid was removed with an alkali phosphatase (made by Takara Shuzo Co., Ltd.). The plasmid DNA and the above-mentioned DNA fragment of about 1.9 kb were mixed, ligated, and introduced into E. coli JM109 (made by Takara Shuzo /15
Co., Ltd.). The plasmid held in the transformant obtained was investigated, and the plasmid containing only one molecule of the above-mentioned fragment of about 1.9 kb was selected and named a plasmid pGL2. The virus particles ascribed to said plasmid are infected on the cells, so that a GFP gene can be integrated onto the chromosomes of the cells being infected. Figure 3 shows a gene region of a retrovirus being integrated into the chromosomes of the cells being infected.

(2) Preparation of vGL2 virus supernatant fluid.

The virus ascribed to the plasmid pGL2 is named a vGL2 virus, and a supernatant fluid containing said virus was prepared using BOSC23 cells (Proc. Natl. Acad. Sci. USA, Vol. 90, pp.8392-8396 (1993)) as packaging cells. In other words, the cells were cultured for one night in 10 ml Dulbecco modified Eagle's base medium (DMEM, made by Iwaki Glass Co., Ltd.) containing 10% fetal calf serum (FCS, made by Dainippon Pharmaceutical Co., Ltd.) containing 1 x $10^7$ pieces of BSC23 cells per one sheet of dish for a gelatin-coated cell culture with a diameter of 10 cm (made by Iwaki Glass Co., Ltd.), 59 units/ml penicillin, and 50 $\mu$g/ml streptomycin (both of them were made by Gibuko Co.) (Also, the DMEM used in the following operation includes 50 units/ml penicillin and 50 $\mu$g/ml streptomycin). The medium was exchanged

with a new medium (10 ml) and transfected with 50 $\mu$g of the above-mentioned plasmid pGL2 by a calcium phosphate method. After 8 h, the medium was exchanged with a fresh medium of 10 ml, and after 24 h, the medium was exchanged with a fresh medium of 5 ml. Furthermore, after containing the culture for 24 h, the cultured supernatant fluid was sampled. The cultured supernatant fluid sampled was filtered by a filter of 0.45 $\mu$ (made by Millipore Co.), so that a vGLS virus supernatant fluid stock was attained. It was held at - 80°C until being used.

(3) Measurement of titer of supernatant fluid

The titer of the supernatant fluid was measured according to a standard method (Journal of Virology, Vol. 62, pp.1120-1124 (1988)) using NIH/3T3 cells (ATCC CRL-1658). In other words, DMEM including 10% calf serum (CS, made by Gibuko Co.) containing 2,000 pieces of NIH/3T3 cells was added to each well of a plate for a tissue culture (made by Iwaki Glass Co., Ltd.) of 6 wells and cultured for one night, and a series-diluted virus /16
supernatant fluid and a hexadimetholin bromide (polyblene: made by Aldrich Co.) with a final concentration of 7.5 $\mu$g/ml were added to each well. It was incubated at 37°C for 24 h, and the medium was exchanged with DMEM containing G418 (made by Gibuko Co.) with a final concentration of 0.75 mg/ml and 10% CS and further continuously incubated. After 10-12 days, the grown G418 resistant colony was dyed with a crystal violet, and the number was recorded. From the value in which the number of colony per well was multiplied by the dilution rate of the virus supernatant

fluid, the number (cfu/ml) of infected particles being included per 1 ml supernatant fluid, that is, the titer of the virus supernatant fluid was calculated, and the amount of virus supernatant fluid being used in the following experiments was determined based on the titer.

(4) Transfer of foreign gene GFP to testicular cells of mice

30 mg/kg busulphan was dosed into the peritoneal cavity of MCH (ICR) male mice of 4 weeks of age in which four mice were one group, and the mice were divided into a group in which a virus was inoculated three times on the 14th day, 17th day, and 20th day after dosing (group 1), a group in which a virus was inoculated three times on the 21st day, 24th day, and 27th day after dosing (group 2), a group in which a virus was inoculated three times on the 28th day, 31st day, and 34th day after dosing (group 3), and a group in which a virus was inoculated three times on the 35th day, 38th day, and 41st day (group 4). The virus was inoculated by a method that inoculated 50 $\mu$l each of the vGL2 virus supernatant fluid of 1 x $10^4$ cfu/ml into both testes. On the 4th day, 11th day, 18th day, and 25th day after the final virus inoculation, the testes were extracted from each one mouse of each group, and a testicular cell sample was prepared. The GFP gene in the cells was detected by the PCR method, and the fluorescence of the GFP gene product was measured by the FACS.

(5) Analysis of GFP gene manifestation in testicular cells

of mice by FACS

The epidermis was removed from the testes of one mouse extracted by Application Example 2(4), and the tissue was disintegrated by a homogenizer. Here, cell bulks were dissociated by adding 1 ml 0.25% trypsin/EDTA solution, and the remaining cell bulks were removed by a mesh of 30 $\mu$, so that a testicular cell sample was prepared. The testicular sample obtained in this manner was provided to the analysis of the FACS, and the manifestation of the GFP gene was investigated. In the analysis, FACVantage was used, and the fluorescence ascribed to the GFP was measured at an excitation wavelength of 488 nm and a measurement wavelength of 515-545 nm. /17

Figure 4 shows the analysis results of the testicular sample on the 25th day after the virus inoculation of the group (3) and the analysis results of a testicular sample of the mouse of the same week of age as a control group in which no virus was inoculated after dosing the busulphan. It was confirmed that in the group in which the virus was inoculated, the region (the region with a relative fluorescence intensity of 30-100 of the GFP indicated on the abscissa of the figure) showing the fluorescence ascribed to the GFP which could not be seen in the control group existed and the GFP gene introduced was manifested in the testicular cells.

(6) Detection of GFP gene introduced into testicular cells of mice

Some of the testicular samples prepared by Application Example 2(5) were selected, and a chromosome DNA was prepared. About 1 x $10^6$ pieces of cells were sampled from the above-mentioned testicular sample and suspended in 700 $\mu$l DNA extracting solution (10 mM tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 39 mM DTT, and 2% SDS). 35 $\mu$l proteinase K (10 mg/ml, made by Merc Co.) was added to the suspension and held at 37°C for one night, and 2 $\mu$l ribonuclease A (20 mg/ml, made by Sigma Co.) was added to it and held at 37°C for 2 h. The reaction was stopped by a phenol chloroform extraction, and the DNA was recovered by an ethanol precipitation and dissolved in 100 $\mu$l TE buffer solution (10 mM tris-HCl (pH 8.0), 1 mM EDTA), so that a chromosome DNA solution was formed.

It was confirmed by the PCR method that the vGL2 virus was infected on the cells and the GFP gene was integrated into the chromosome. A PCR reaction containing the above-mentioned chromosome DNA and oligonucleotides GFP-13 and GFP-16R (the base sequence of the oligonucleotides GFP-13 and GFP-16R are shown in sequence Nos. 1 and 2 of the sequence table) synthesized based on the sequence of the GFP gene was prepared, treated at 94°C for 1 min, and reacted for 30 cycles in which 94°C for 30 sec - 63°C for 30 sec - 72°C for 30 sec was 1 cycle. Also, using TaKaPa Taq (made by Takara Shuzo Co., Ltd.) as the PCR and an attached buffer solution for reaction, a reaction solution was prepared. Also, chromosome DNA solutions prepared from the NIH/3T3 cells infected with vGL2 virus and a testicular cell sample of a mouse

at the same week of age in which no virus was inoculated after dosing the busulphan by an operation similar to the above-mentioned case were used as a positive control and a negative control, respectively. /18

As a result, as shown in Table V, for the chromosome DNAs prepared from the testicular cells on the 18th day after the final virus inoculation of the group 2, on the 4th and 25th days after the final virus inoculation of the group 3, and on the 4th and 18th days after the final virus inoculation of the group 4, an amplification of the DNA fragments of 234 bp ascribed to the GFP gene was recognized.

From the above results, it was shown that in the groups in which the virus was inoculated from the fourth week to the fifth week or from the fifth week to the sixth week after the busulphan dosage, the foreign gene, that is, the GFP gene was integrated with high efficiency onto the chromosomes. Therefore, it is clarified that it is important to inoculate the virus several times around the fifth week after the busulphan dosage which is a recovery timing of the reduced testicular cells which was clarified from the preliminary experiment shown in Application Example 1.

The results of Application Example 2(4)-(6) show that in an in vivo integration of an intended gene into chromosomes of the testicular cells, it is important to transfer the intended gene to a vector having an integration power and to inoculate it at the timing that the testicular cells are recovered from the

obstacle of a drug.

Table V

Detection of GFP gene by PCR

| 投与群 | ウイルス最終接種後日数 | PCR検定結果 |
|---|---|---|
| グループ1 | 18 | − |
| グループ2 | 11 | − |
| | 18 | + |
| グループ3 | 4 | + |
| | 11 | − |
| | 18 | − |
| | 25 | + |
| グループ4 | 4 | + |
| | 11 | − |
| | 18 | + |
| ポジティブコントロール | | + |
| ネガティブコントロール | | − |

+：234 bp 断片の増幅あり

−：234 bp 断片の増幅なし

1. Dosage group
2. Number of days after the final virus inoculation
3. PCR test results
4. Group 1
5. Group 2
6. Group 3
7. Group 4
8. Positive control
9. Negative control
10. +: Amplification of 234 bp fragment

+ No amplification of 234 bp fragment

Application Example 3

Transfer of GFP gene to testicular cells of the epididymis of mice and manifestation

(1) Transfer of GFP gene to testicular cells of the epididymis of mice

30 mg/kg busulphan was dosed into the peritoneal cavity of 16 MCH (ICR) male mice of 4 weeks of age, and 50 $\mu$l each of vGL 2 virus supernatant fluid of 1 x $10^4$ cfu/ml was inoculated into both testes on the 28th day, 31st day, and 34th day after dosing. On the 28th day, 35th day, 42nd day, and 49th day after the virus inoculation, the epididymis was extracted from four mice each, and a sperm fraction was prepared. The fluorescence of the GFP gene product was measured by the FACS, the fluorescence-positive fraction cells were collected, and the GFP gene was detected in the fluorescence-positive fraction cells by the PCR method.

(2) Analysis of GFP gene manifestation in testicular cells in the epididymis of mice

The epididymis of the mice described in Application Example 3(1) was finely cut in 2% penicillin/streptomycin by scissors, and cell bulks were removed by a mesh of 30 $\mu$, so that a sperm fraction was prepared. For the sperm fraction, according to the method described in Application Example 2(5), the GFP gene manifestation was confirmed by the FACS. Also, at that time, the fluorescence ascribed to the GFP separated a positive cell

fraction.

Figure 5 shows the analysis results of the sperm fraction ascribed to the mice on the 42nd day after the virus inoculation and the analysis results of a testicular sample of the mouse of the same week of age as a control group in which no virus was inoculated after dosing the busulphan. As shown in the figure, it could be confirmed that the sperms (the region with a relative fluorescence intensity of 8-20 of the GFP indicated on the abscissa of the figure) for manifesting the GFP in the epididymis of the mice on the 42nd day after the virus inoculation existed

(3) Detection of GFP gene of testicular cells in the epididymis of mice

About 1 x $10^5$ pieces of cells were sampled from the sperm fraction positive to the GFP fluorescence separated by the FACS in Application Example 3(2) and suspended in 700 $\mu$l DNA extracting solution (10 mM tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 39 mM DTT, and 2% SDS). 35 $\mu$l proteinase K (10 mg/ml) was added to the suspension and held at 37°C for one night, and 2 $\mu$l ribonuclease A (20 mg/ml) was added to it and held at 37°C for 2 h. The reaction was stopped by a phenol chloroform extraction, and the DNA was recovered by an ethanol precipitation and dissolved in 30 $\mu$l TE buffer solution, so that a chromosome DNA solution was formed.

For the chromosome DNA solution, the GFP gene was detected by the method described in Application Example 2(5). As a result, as shown in Table VI, an amplification of 234 bp DNA

fragment showing the existence of the GFP gene was recognized for the chromosome DNA prepared from the sperms on the 42nd and 49th days after the virus dosage.

Table VI /21

Detection of GFP gene by PCR

| ウイルス最終投与後日数 | ウイルス未投与群 | ウイルス投与群 |
|---|---|---|
| 28日目 | − | − |
| 35日目 | − | − |
| 42日目 | − | + |
| 49日目 | − | + |

+：234 bp 断片の増幅あり

−：234 bp 断片の増幅なし

1. Number of days after the final virus dosage
2. Group without dosing the virus
3. Virus-dosed group
4. 28th day
5. 35th day
6. 42nd day
7. 49th day
8. +: Amplification of 234 bp fragment

   + No amplification of 234 bp fragment

Claims 

1. A method for transferring a gene to testicular cells of a vertebrate, characterized by including a process that reduces the number of testicular cells and a process that inoculates a recombinant virus having a foreign gene into testes.

2. The method for transferring a gene of Claim 1, characterized by the fact that the process for reducing the number of testicular cells is carried out by a method being selected from chemical method, physical method, and biological method.

3. The method for transferring a gene of Claim 2, characterized by the fact that an alkylating agent is used.

4. The method for transferring a gene of any of Claims 1-3, characterized by the fact that the recombinant virus is inoculated into the testes at a recovery stage of the testicular cells.

5. The method for transferring a gene of any of Claims 1-4, characterized by the fact that a recombinant virus having a chromosome integration power is inoculated.

6. The method for transferring a gene of Claim 5, characterized by the fact that the recombinant virus uses a virus vector deficient for replicability.

7. The method for transferring a gene of Claim 5 or 6, characterized by the fact that a recombinant retrovirus is inoculated.

8. A vertebrate, characterized by the fact that a foreign gene is transferred to the testicular cells by the method of any of

Claims 1-7.

9. Testicular cells to which a foreign gene is transferred, characterized by being obtained from the vertebrate of Claim 8.

10. The testicular cells of Claim 9, characterized by being selected from spermatogonia, primary spermatocytes, spermatids, and sperms.

11. A vertebrate to which a foreign gene is transferred, characterized by being obtained by mating of the vertebrate of Claim 8.

12. A vertebrate to which a foreign gene is transferred, characterized by being obtained from an artificial egg artificially prepared using the testicular cells of Claim 9 or 10.

Figure 1:

1. Number of cell

2. Relative fluorescence intensity

3. Apoptosis

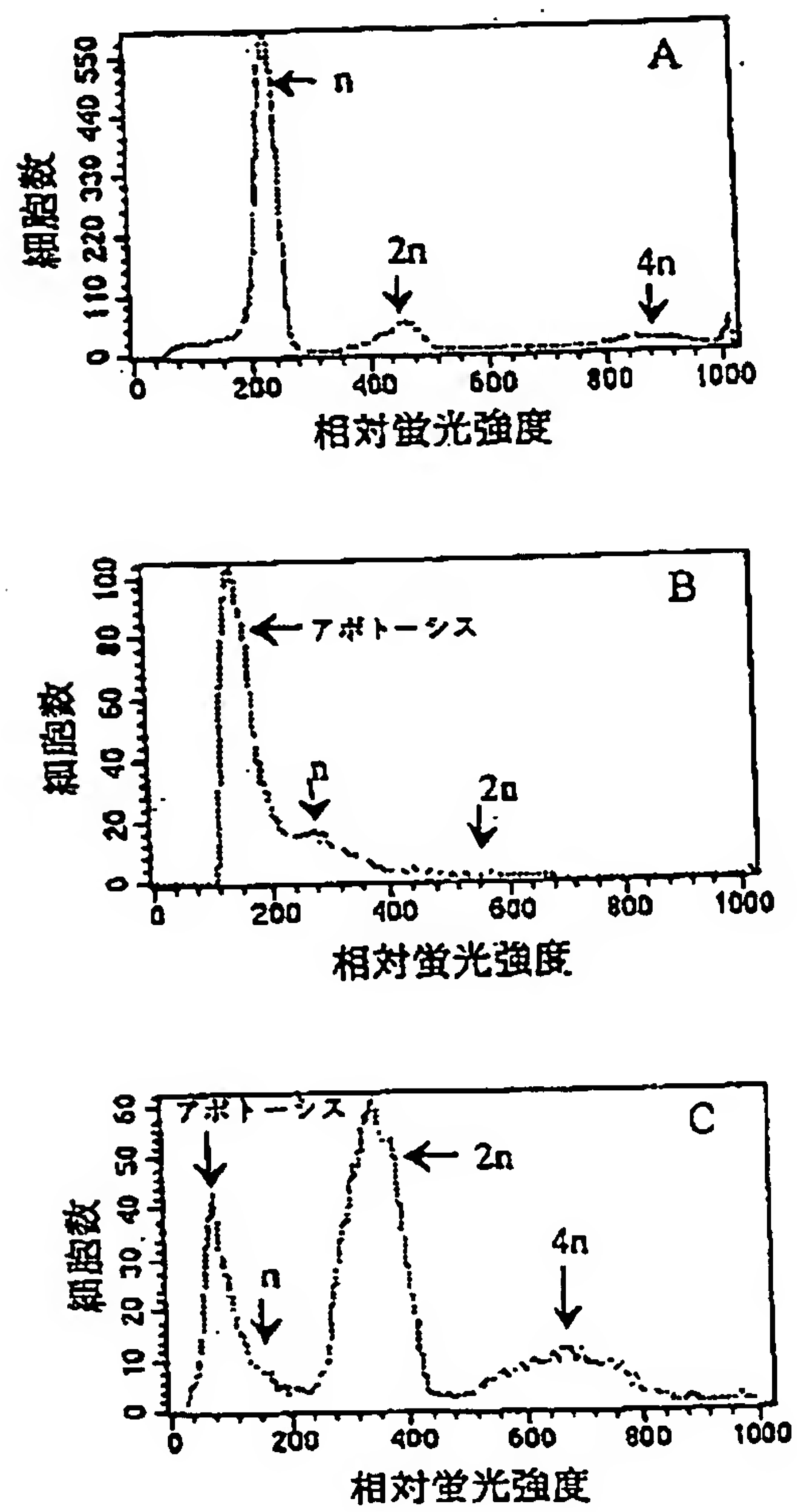


Figure 2:

1. Pregnancy rate (%)

2. Time (weeks) after dosing the drug

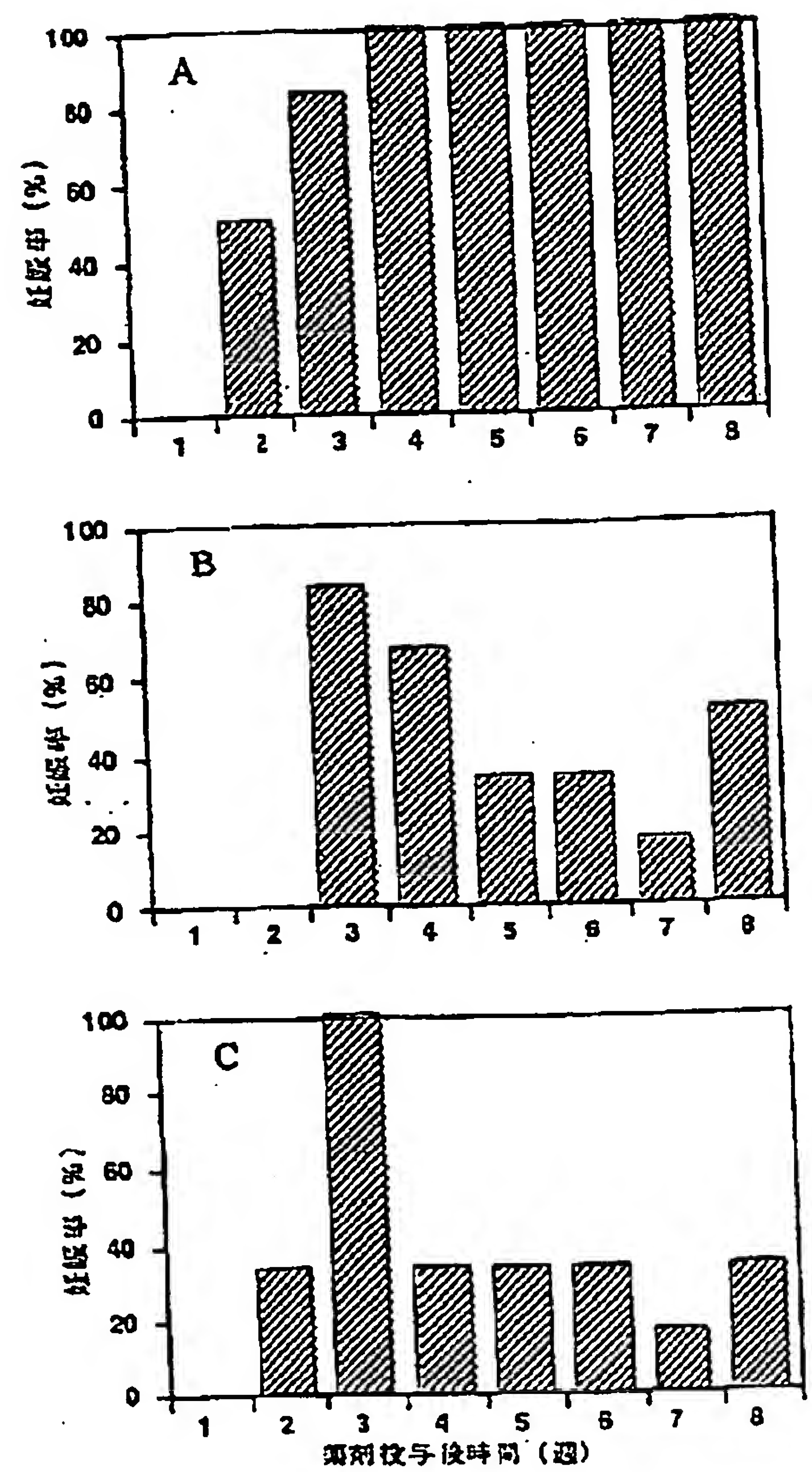


Figure 3

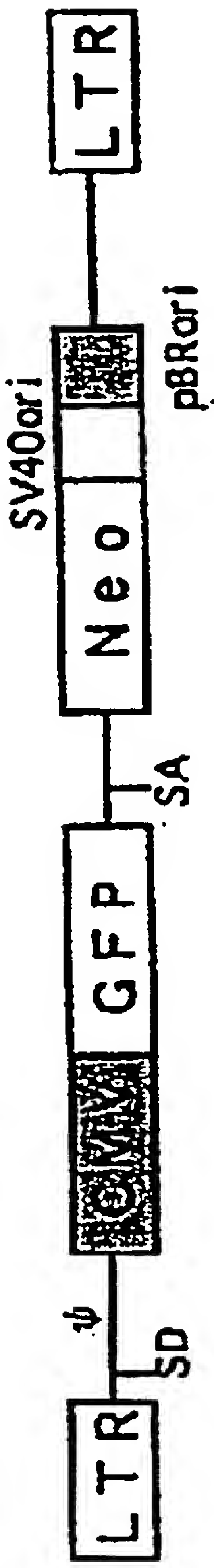


Figure 4:

1. Number of cell

2. Relative fluorescence intensity

3. Virus inoculation

4. Control group

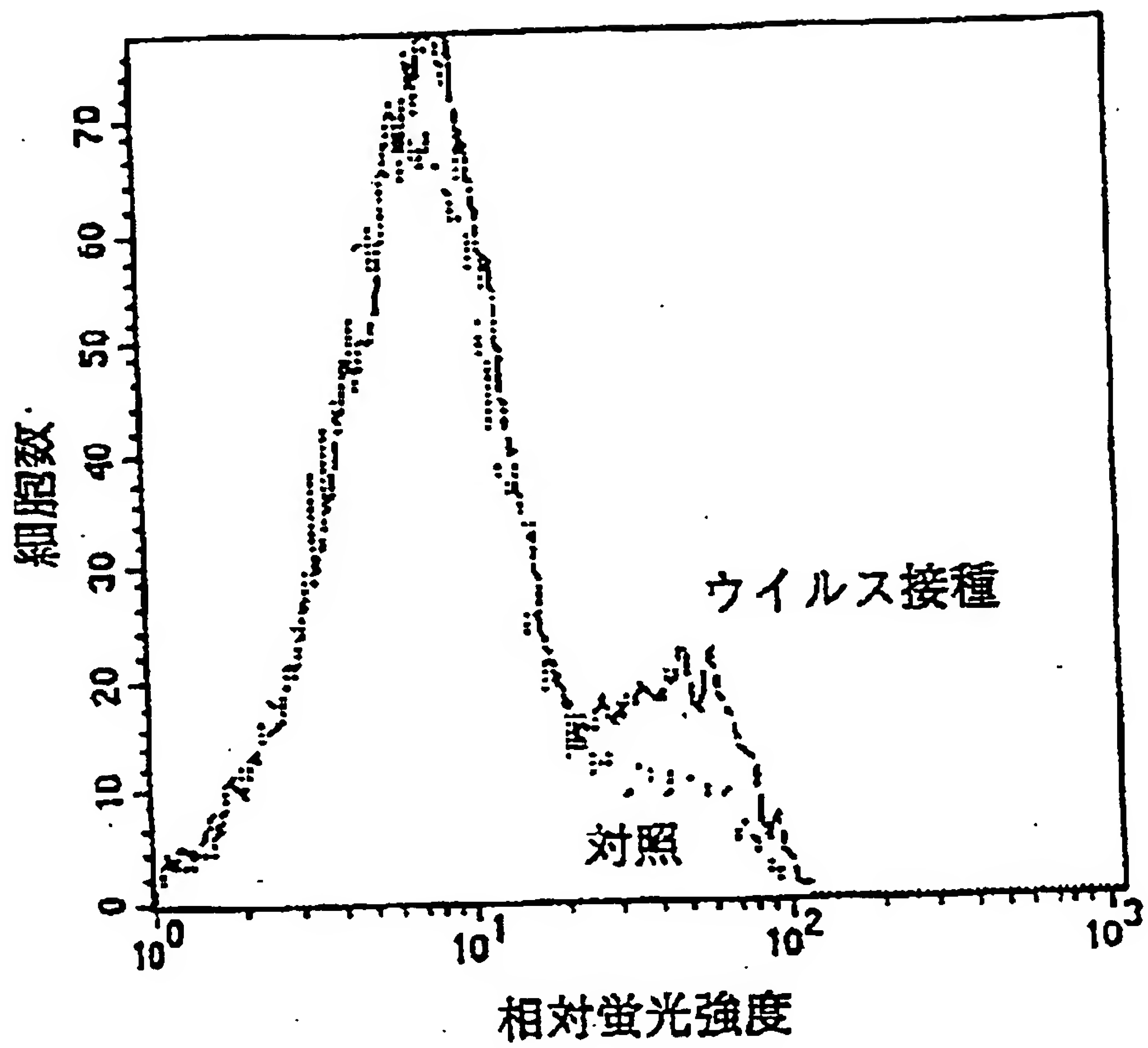
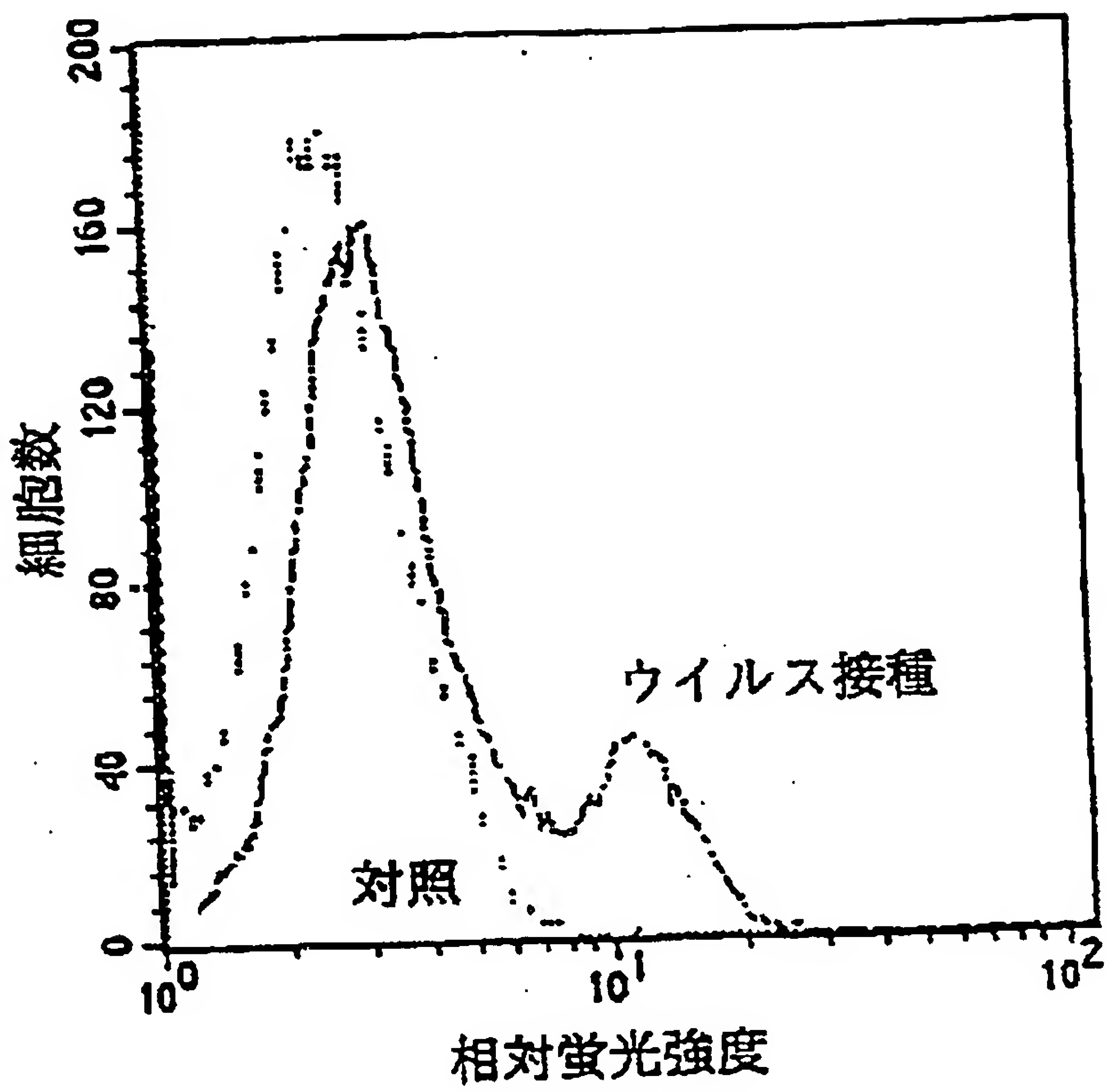


Figure 5:

1. Number of cell
2. Relative fluorescence intensity
3. Virus inoculation
4. Control group



Sequence table

<120> Method for transferring a gene to germ cells

〈110〉 寶酒造株式会社 他

Taxara Shuzo Co., Ltd. et al.

〈120〉 生殖細胞への遺伝子導入法

〈130〉 661149

〈150〉 JP 10-15629

〈151〉 1998-1-28

〈160〉 2

〈210〉 1

〈211〉 22

〈212〉 DNA

〈213〉 Artificial Sequence

〈400〉 1

catcatggcc gacaagcaaa ag 22

〈210〉 2

〈211〉 21

〈212〉 DNA

〈213〉 Artificial Sequence

〈400〉 2

atcccagcag cggtcacaaa c 21